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Poumay, Yves; Boucher, F.; Smekens, Michèle; Degen, Annie; Leloup, Robert

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Basal Cell Adhesion to a Culture Substratum Controls the Polarized Spatial Organization of Human Epidermal Keratinocytes into Proliferating Basal and Terminally Differentiating Suprabasal Populations

Y. Poumay, F. Boucher, M. Leclercq-Smekens, A. Degen and R. Leloup
Département d'Histologie-Embryologie, Facultés Universitaires Notre-Dame de la Paix, Belgium

Abstract. The contribution of adhesion to an extracellular matrix in the polarized spatial organization of keratinocytes was studied in dispase-detached cultures stored as floating sheets. Proliferating and terminally differentiating cell populations were, therefore, localized on tissue sections by their DNA-synthesizing ability and involucrin immunostaining, respectively. A progressive reorganization was induced from superposed proliferating and differentiating layers into clusters exhibiting differentiating cells on the outside. Measurements of proliferation and terminal differentiation in detached cultures revealed the progressive disappearance of proliferating cells, followed by an increase in involucrin-positive cells. Attempts to block the spatial reorganization by the addition of components of the extracellular matrix remained unsuccessful. These results suggest that basal anchorage is responsible for the superposition of proliferating and differentiating cells in keratinocyte cultures. They afford new arguments for the induction of terminal differentiation in non-adhesive keratinocytes which exhibit a concomitant modification of cell shape.

Keywords: Cell adhesion; Keratinocytes; Proliferation; Spatial organization; Terminal differentiation

Correspondence and offprint requests to: Yves Poumay, Département d'Histologie-Embryologie, Facultés Universitaires Notre-Dame de la Paix, 61, Rue de Bruxelles, B-5000 Namur, Belgium. Fax: 81 23 03 91.

Introduction

The epidermis is a highly polarized tissue. DNA synthesis and mitosis occur in cells localized in the basal layer, whilst the onset of terminal differentiation is observed in cells moving through the upper layers. The progressive acquisition of a terminally differentiated phenotype includes the synthesis of different keratin subtypes, synthesis of involucrin (a precursor of the cornified envelope), the production of lipid-containing membrane-coating granules and the synthesis of filaggrin and loricrin. Ultimately, this process produces an impermeable, insoluble protective layer in the outermost layer of the epidermis which is composed of cells in the final stage of differentiation (where the assembly of the cornified envelopes is completed) (Fuchs 1990).

Cultures of human epidermal keratinocytes have been shown to mimic many of the characteristic features of their original tissue, including its polarized spatial organization (Green 1980; Watt 1988). Basal cells adhering to the culture substratum represent the proliferating population of keratinocytes as DNA synthesis is restricted to this basal layer (Rheinwald and Green 1975; Dover and Potten 1983; Poumay et al. 1991). Moreover, these basal cells are nearly always free of detectable involucrin since in stratified cultures the onset of involucrin synthesis is associated with cells of the suprabasal layers which undergo terminal differentiation (Rice and Green 1979; Banks-Schlegel and Green 1981; Dover and Watt 1987). Cultures of keratinocytes have, in consequence, been used for the study of the control of proliferation and terminal differentiation. Initially, suspensions of keratinocytes in methylcellulose enabled these studies to be carried out (Green

1977). Suspended keratinocytes stop synthesizing DNA and their terminal differentiation is induced, as revealed by involucrin synthesis (Watt et al. 1988). Thus, it is thought that loss of contact with other keratinocytes and the substratum triggers a change in the morphology of these cells (suspended keratinocytes have a round shape) and so leads to terminal differentiation (Watt et al. 1988). It is interesting to note that the binding of an extracellular matrix protein (fibronectin) to its receptor seems to regulate the differentiated gene expression in these cells (Adams and Watt 1989).

Undissociated sheets of cultured keratinocytes can be detached from the culture substratum by dispase treatment and are then suitable for grafting (Green et al. 1979). These sheets retract rapidly and basal cells subsequently exhibit a more cuboidal morphology, maintaining contact with neighbouring and suprabasal cells (Banks-Schlegel and Green 1981). We recently studied the storage of these sheets and observed a progressive, irreversible inhibition of the proliferative activity (DNA synthesis) of keratinocytes (Poumay et al. 1991; Boucher et al. 1991). This inhibition is almost complete within 12–16 h after dispase detachment, which correlates well with the observations of keratinocytes in suspension (Adams and Watt 1989). These results suggest that detachment of cells from the substratum and/or the loss of the squamous morphology could trigger terminal differentiation in keratinocyte sheets as it does in suspended cells, although cell–cell interaction was preserved in the former situation.

Here we have studied proliferating and terminally differentiating populations in dispase-detached cultured epidermal sheets and observed the spatial organization of keratinocytes in these conditions. Our observations provide new evidence that the adhesion of basal cells to a substratum is necessary to maintain their high proliferative activity. Moreover, storage of dispase-detached cultures causes progressive loss of the characteristic polarity of the cultured stratified epithelium. Detachment of cells from the substratum allows complex cell–cell interactions to occur and permits the reorganization of the proliferating and differentiating cell populations from superposed layers into almost globular clusters. Interestingly, these structures are very similar to those obtained by the reaggregation of suspended single keratinocytes in an agarose pellet (Watt 1984). Finally, we present evidence that terminal differentiation is also induced in these conditions.

Materials and Methods

Culture of Human Keratinocytes

Human keratinocytes isolated from adult skin epidermis obtained after plastic surgery were cultured, according to the method of Rheinwald and Green (1975), in the presence of a feeder layer of mitomycin C-treated 3T3 cells, in culture medium composed of one part Ham's F12 and three parts Dulbecco's modified Eagle's medium. This was supplemented with 10% fetal calf serum, 5 μg/ml insulin, 0.4 μg/ml hydrocortisone, 10^{-10} mol/l cholera toxin, 5 μg/ml transferrin, 2×10^{-9} mol/l triiodothyronine, 1.8×10^{-4} mol/l adenine and 10 ng/ml epidermal growth factor. Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere and the medium was changed ever 2–3 days. At confluence, the cultures were trypsinized with a solution of trypsin (0.1%) and EDTA (0.02%) and the cells stored frozen in liquid nitrogen using standard methods. When required, keratinocytes were thawed and Multi 6-well culture plates (Falcon) were inoculated with 1×10^5 cells per dish in the presence of the same feeder layer. Confluent secondary cultures were used throughout the study.

Detachment and Storage of Cultured Keratinocyte Sheets

The cultures were treated with dispase II 0.25% (w/v) in a serum-free medium, according to the method of Green et al. (1979), for about 1 h at 37 °C. It is interesting to note that the phenomena described below in 'Results' can be reproduced with cultures treated in the same way with dispase, but in serum-containing medium. After detachment the keratinocyte sheets were rinsed twice in phosphate-buffered saline (PBS) and then stored in culture medium at 37 °C for 0 to 48 h. For the measurements of sheet diameters, triplicate cultures were photographed at different time intervals during their contraction and the maximum diameter was worked out on prints, using the diameter of the culture dish in order to calculate the magnification. In some experiments components of the extracellular matrix were added to the storage medium at a final concentration of 100 μg/ml, according to Adams and Watt (1989). Human plasma fibronectin and mouse EHS laminim were purchased from Biomedical Technologies (Stoughton, MA), bovine collagen type I (Vitrogen 100) from Collagen Corporation (Palo Alto, CA) and basement membrane Matrigel from Flow Laboratories (Brussels, Belgium).

Tissue Fixation, Sectioning and Staining

Cultures obtained after detachment and stored for different periods were then fixed by 3.7% formaldehyde in PBS at room temperature for 30 min, dehydrated in methanol and embedded in paraffin wax. Sections (6 μm) were prepared perpendicular to the culture plane (Watt 1984) in different regions of the central area of detached sheets (the curled edge of cells formerly on the wall of the dish (Green et al. 1979) was usually cut off before embedding). For histological observations, sections were stained with haemalum and erythrosin, mounted in DPX medium (BDH, Poole, England) and photographed with a Zeiss Photomicroscope III.

Dual-Label Immunofluorescence of DNA-Incorporated 5′-Bromo-2′-deoxyuridine (BrdUrd) and Involucrin

Cultured keratinocyte sheets utilized for DNA synthesis studies were incubated in culture medium containing 10 μmol/l BrdUrd (Sigma, Saint-Louis, MO) for 1 h at 37 °C before formaldehyde fixation and tissue processing as described above. For immunofluorescence studies, sections were rehydrated and pre-treated with pepsin, as recommended, to enhance the immunoreactivity of anti-BrdUrd on tissue fixed with agents capable of cross-linking effects on DNA (Schutte et al. 1987). The cellular DNA was then denatured by incubation in 2 mol/l hydrochloric acid (30 min, 37 °C) and neutralized with two washes in 0.1 mol/l sodium tetraborate (pH 8.5). Sections were further rinsed twice in PBS containing Tween 20 (0.5%) and then covered for 30 min in a humidified chamber with a mixture of an anti-BrdUrd monoclonal antibody (Becton Dickinson, Mountain View, CA) at a 1 : 10 dilution and an anti-involucrin rabbit antiserum (generous gift from F.M. Watt, Imperial Cancer Research Fund, London) (Dover and Watt 1987) at a 1 : 250 dilution. After two washes in PBS–Tween 20 the sections were incubated in the same conditions in a mixture of second antibodies: a Texas Red-conjugated goat antibody to mouse antibody (Molecular Probes, Eugene, OR) at a 1 : 20 dilution and a FITC-conjugated swine antibody to rabbit antibody (Dakopatts, Denmark) at a 1 : 20 dilution. Finally, the sections were washed twice in PBS–Tween 20 and mounted in Glycergel (Dakopatts). Sections were examined using a Zeiss Photomicroscope, equipped with a RS-III epicondensor, and Texas Red- and fluorescein-specific filters and dichroic mirrors.

Measurements of Cell Proliferation

In order to determine the labelling index on culture sections (percentage of DNA-synthesizing nuclei), detached sheets were processed as described by Poumay et al. (1991). Briefly, BrdUrd-containing nuclei were immunolabelled using as a fluorescent second antibody a FITC-conjugated F(ab′)2 fragment of rabbit antibody to mouse antibody (Dakopatts), whilst all nuclei were lightly stained with propidium iodide (Sigma). At least 500 nuclei were scored per culture sheet. The efficiency of colony formation was determined by the method of Rheinwald and Green (1975).

Determination of the Percentage of Involucrin-Positive Cells and of Cornified Envelopes

At intervals after dispase detachment, epidermal cells were harvested from stored quadruplicate sheets in 0.1% trypsin and 0.02% EDTA and counted using a haemocytometer. For involucrin scoring the method used was the one described by Read and Watt (1988). Trypsinized cells were resuspended in culture medium at a concentration of 10^6 cells/ml and 50 μl of the suspension air dried on round glass coverslips at 37 °C for 30 min. The cells were then fixed by placing the coverslips in 3.7% formaldehyde in PBS for 15 min at room temperature and permeabilized using a 5 min incubation in absolute methanol, pre-chilled on ice. Staining with the anti-involucrin rabbit antiserum was carried out at room temperature for 45 min at a 1 : 250 dilution in PBS followed by FITC-conjugated swine antibody to rabbit antibody (Dakopatts, 1 : 20). Coverslips were mounted and examined as above. Several fields, selected at random, were photographed twice, once using epifluorescence microscopy for the involucrin staining and once using phase-contrast microscopy for determining the total number of cells. At least 300 cells were scored per sheet to calculate the percentage of involucrin-positive cells.

The cornified envelope content of keratinocytes, suspended by trypsinization of stored detached cultures, was determined according to Rice and Green (1979). Briefly, one aliquot of keratinocytes was pelleted by centrifugation and then dissolved immediately by boiling in a 2% SDS/20 mmol/l dithiotreitol solution for 5 min at 100 °C. The envelopes were then counted with a haemocytometer using phase-contrast microscopy.

Results

Previous investigations into the use of dispase for the detachment of confluent keratinocyte cultures have already revealed that, upon detachment, a rapid contraction of the culture diameter occurs with a concomitant rapid change in basal cell shape from a flattened to a more columnar morphology (Green et al. 1979; Banks-Schlegel and Green 1981). At the same time, the spatial organization of the cultured epidermis is unchanged by the detachment: tissue sections exhibit one layer of columnar cells (basal phenotype) covered by one to three layers of larger flattened cells (suprabasal phenotype) (Fig. 1A). After several hours of storage of these detached cultured sheets in culture medium before tissue fixation and processing, we observed, following the rapid initial modification, that their contraction continued slowly, as indicated by the measurement of sheet diameters (Fig. 2). Interestingly, histological sections in different regions of the slightly puckered central area of replicate detached sheets revealed a pronounced rearrangement of cultured keratinocytes with a progressive stacking of cells exhibiting the basal phenotype. In consequence, this novel spatial organization produced an enhanced thickness of the cultured sheets less than 6 h after detachment (Fig. 1B). Trypsinization of sheets and cell counting with a haemocytometer revealed that this culture thickening could not have been the result of cell division. When storage was prolonged, the contraction of cultures continued (Fig. 2), their rigidity increased and the keratinocytes exhibited a globular clustering with squamous cells becoming progressively localized at the previous basal side of the sheets and more polygonal keratinocytes localizing

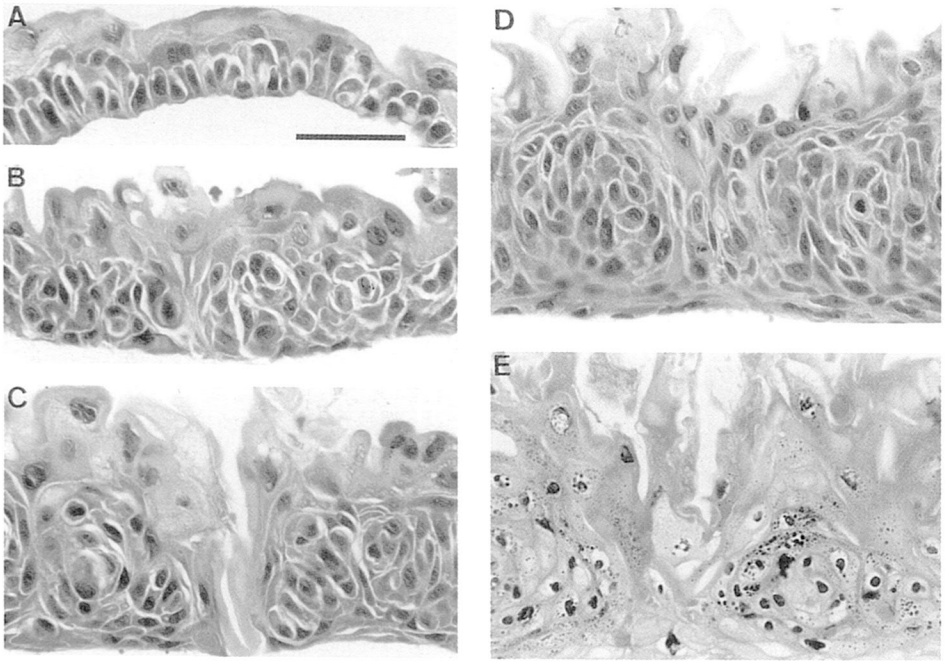

Fig. 1A–E. Dispase-detached epidermal sheets formed by cultured keratinocytes. Cultures were fixed with formaldehyde immediately (0 h) after detachment (**A**) or stored at 37 °C in culture medium of 6 h (**B**), 14 h (**C**), 24 h (**D**) or 48 h (**E**) before fixation. They were then embedded in paraffin and sectioned before staining with haemalum and erythrosin. In **A**, due to rapid retraction, the basal cells exhibit a columnar morphology. Then clustering progressively occurs with a concomitant thickening of sheets (**B–D**). Note that 48 h after detachment most cells exhibit the differentiated phenotype or degenerate (**E**). Scale bar represents 50 μm.

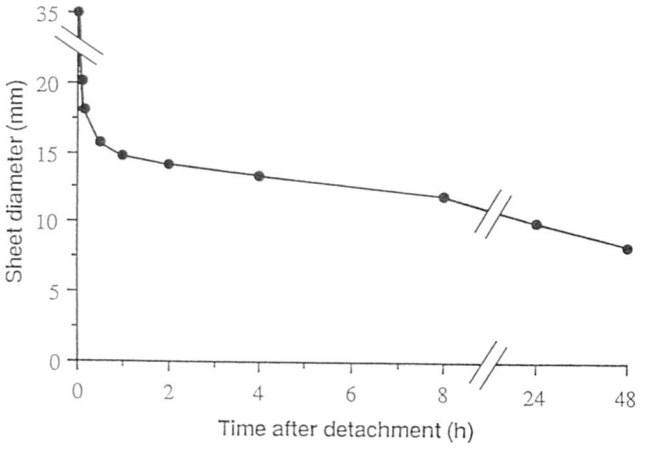

Fig. 2. Diameter of dispase-detached epidermal sheets (cultured in 35 mm diameter dishes) after different storage periods. Each point represents the mean diameter of two sheets processed simultaneously.

at the centre of clusters (Fig. 1B–D). It occasionally happened that typical suprabasal cells surrounding these clusters joined both sides of the sheets and this spatial reorganization progressively produced a hill and valley appearance on the previous apical side of the culture, whilst the basal side remained fairly smooth. Lastly, when the cultures had been stored for 48 h after dispase detachment, the suprabasal phenotype predominated and most clusters exhibited some degenerating cells (Fig. 1E).

In order to follow the relative movements of basal and suprabasal cells, dual-label immunofluorescence was performed on culture sections: the proliferative activity of basal cells was revealed by a 1 h incorporation of BrdUrd into cells that had entered the S phase of DNA synthesis during incubation with this thymidine analogue (this labelling procedure allows only a fraction of proliferating cells to be identified); simultaneously each cell that had entered the process of terminal differentiation was identified by the labelling of involucrin. The most characteristic steps of the reorganization phenomenon are shown in Fig. 3, which illustrates sheets fixed either immediately after detachment by dispase treatment or 6, 14 and 24 h later. BrdUrd immunolabelling (Fig. 3B, D, F, H) proved that proliferating keratinocytes, originally located in the basal layer, migrated upwards into clusters during storage of the cultures. At the same time, the determination of the labelling index, coupled with the measurement of colony-forming efficiency after trypsinization of the stored detached sheets, confirmed our previous observations that cellular proliferation was progressively and irreversibly inhibited in cultures separated from the substratum (Poumay et al. 1991; Boucher et al. 1991). A rapid decrease in the labelling index was in fact observed, especially within the first 10 h after the detachment of cultures. Only occasional BrdUrd-positive nuclei were still observed more than 12 h after detachment (their number is unusually high in Fig. 3F, which has been chosen to illustrate their localization), and practically no BrdUrd-positive nucleus could still be detected more than 18 h after detachment (Figs. 3H and 4A). In order to check that the accessibility of BrdUrd to proliferating cells is not impeded by the surrounding differentiating keratinocytes, the labelled index of anchored cultures was compared and found to be identical to that of dispase-treated cultures incubated with BrdUrd immediately after detachment. In addition to these labelling index measurements we determined the capacity of the keratinocytes enclosed in the detached sheets

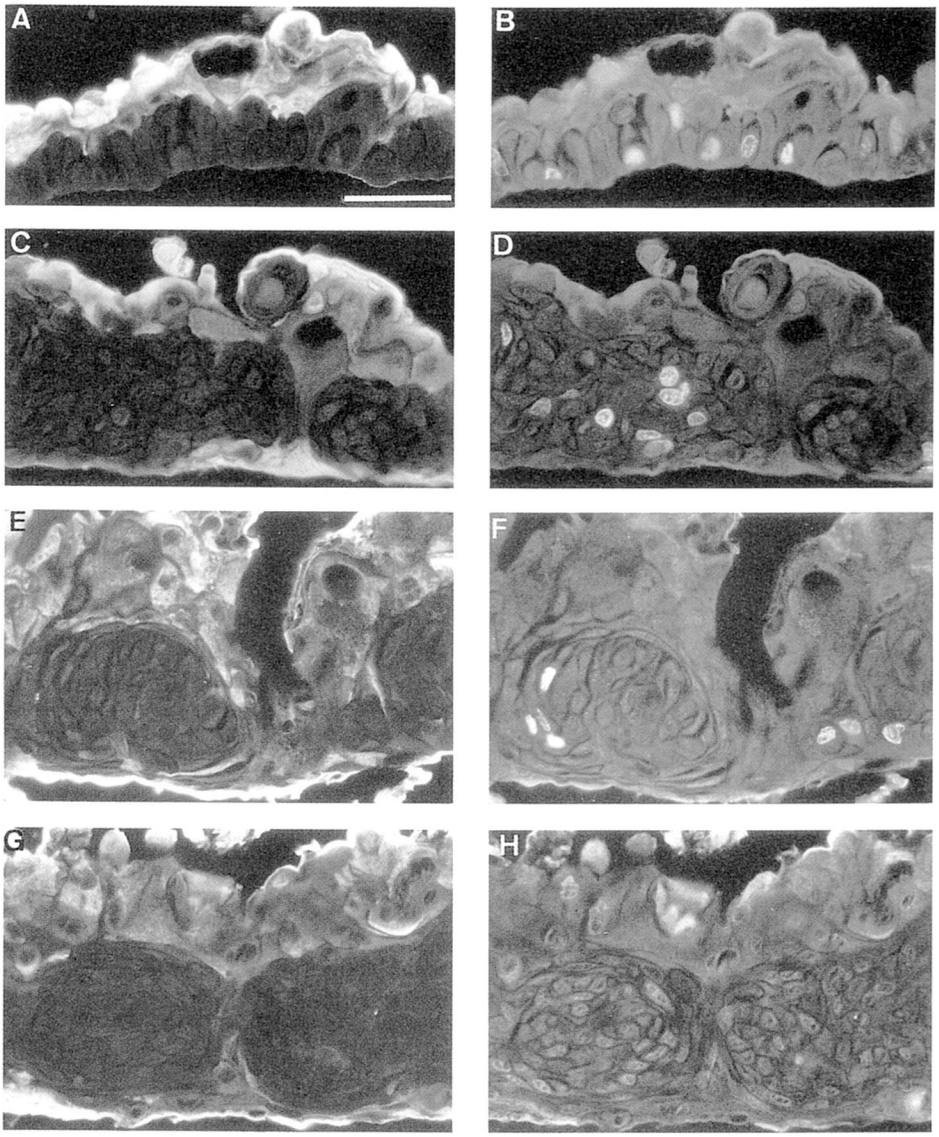

Fig. 3A–H. Dispase-detached epidermal sheets formed by cultured keratinocytes. Cultures were fixed with formaldehyde immediately (0 h) after detachment (**A**, **B**) or stored at 37 °C in culture medium for 6 h (**C**, **D**), 14 h (**E**, **F**) or 24 h (**G**, **H**) before fixation. Each culture has been incubated for 1 h with BrdUrd-containing medium before fixation. After embedding and sectioning, dual immunolabelling was performed to compare, on the same microscopic fields, fluorescein staining using a rabbit antiserum raised against involucrin (**A**, **C**, **E**, **G**) with Texas Red staining using a monoclonal antibody against BrdUrd (**B**, **D**, **F**, **H**). Background staining is relatively high with Texas Red staining. Scale bar represents 50 μm.

to reinitiate the formation of colonies (colony-forming efficiency is expressed as the percentage of replated cells that are able to produce new colonies). This potential decreases less rapidly than do the labelling indices: colony-forming efficiency is around 12% for keratinocytes dissociated and replated immediately after dispase detachment (0 h), and still about 5% and 3% for cultures processed respectively 16 h or 24 h after detachment, but cultures maintained detached for 48 h contained only very few cells able to form colonies (<0.3%) (Fig. 4B). Moreover, at the same time, the recovered colonies changed progressively from colonies characteristic of meroclones into smaller colonies typical of the slower growing paraclones, according to the terminology of Barrandon and Green (1987a) (data not shown).

The complementary involucrin immunolabelling (Fig. 3A, C, E, G), compared with the BrdUrd immunolabelling, clearly illustrated that proliferating cells are preferentially undifferentiated cells and particularly demonstrated that clusters are formed of undifferentiated keratinocytes which are localized in the middle and surrounded by terminally differentiating cells. Interestingly, cells with a flattened morphology that were located at the basal side of clusters became progressively involucrin-prositive. In the experiments illustrated in Fig. 5 we prolonged the storage period and compared the labelling of involucrin immediately after detachment of the keratinocyte sheets with that obtained 24 h and 48 h later. On the one hand these results clearly show that the formation of clusters was achieved within 24 h, whereas involucrin-positive cells appeared in the centre of clusters between 24 and 48 h after detachment. On the other hand they reveal that, following the inhibition of proliferation, terminal differentiation was induced in those keratinocytes.

The induced differentiation was confirmed by determining the proportion of involucrin-positive cells and the cornified envelope content in the same conditions (Fig. 6). Using these quantitative approaches a signifi-

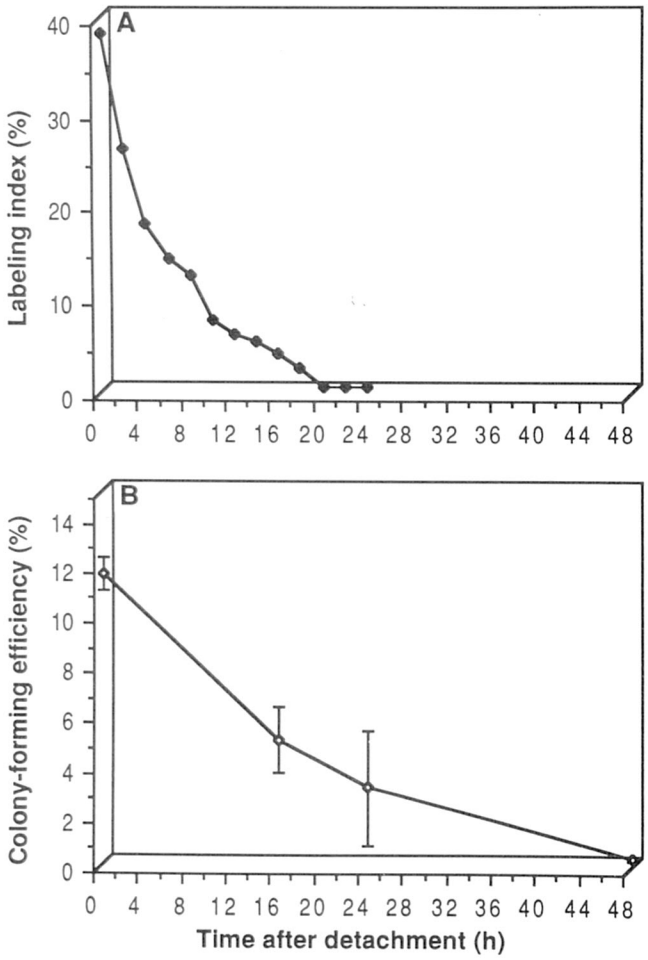

Fig. 4. **A** Labelling index determination using BrdUrd incorporation in keratinocytes of dispase-detached cultured epidermal sheets after different storage periods. **B** Colony-forming efficiency of keratinocytes from dispase-detached cultures. For each time tested, results are means ± SE of colony-forming efficiencies measured in triplicates in each of three different detached cultures.

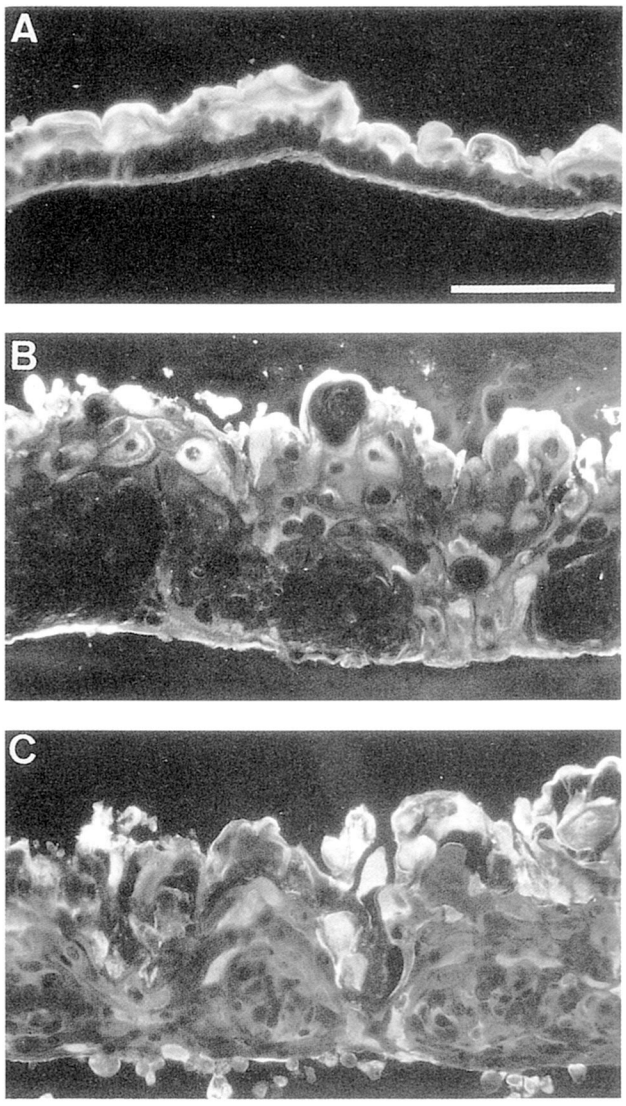

Fig. 5A–C. Immunofluorescent staining of involucrin in dispase-detached cultures of epidermal keratinocytes fixed with formaldehyde immediately after detachment (**A**) or stored at 37 °C in culture medium for 24 h (**B**) or 48 h (**C**) before fixation. Fixed sheets were then embedded in paraffin wax, sectioned and then immunostained with fluorescein using a rabbit antiserum raised against involucrin. Scale bar represents 100 μm.

cant progressive stimulation of terminal differentiation was confirmed when sheets were analysed immediately after dispase detachment and compared with sheets kept detached for 24 h or 48 h before processing. Moreover, the trypsinization of sheets allowed us to study the individual morphology of their component cells. Thus, we observed that cells obtained by immediate trypsinization of detached sheets had a spherical morphology, with involucrin expressed in 20%–25% of the keratinocytes (mainly in the largest cells of the population) (Fig. 7A). These results are in exact agreement with previous studies published by others (Watt and Green 1981; Watt et al. 1988; Adams and Watt 1990). Twenty-four hours after detachment involucrin was still found in these largest cells, but most of them exhibited an irregular shape which was also observed 48 h after detachment (Fig. 7B, C). As spherical envelopes are formed only in keratinocytes cultured in suspension (Green 1980), this unusual non-spherical morphology of isolated cells is likely to correspond to an enhanced formation of cornified envelopes in the epidermal cells, before trypsinization of cultured sheets, in accordance with the quantitative study illustrated in Fig. 6. In addition to this phenomenon, 48 h after dispase detachment of the cultured sheets we noted that a greater number of small keratinocytes became involucrin-positive (Fig. 7C).

Other evidence of a triggered terminal differentiation accompanying the spatial reorganization of keratinocytes was provided by electron microscopic analysis and by [^{35}S]methionine incorporation and polyacrylamide gel electrophoresis (PAGE) analysis of cultured keratinocyte sheets either kept anchored or dispase-detached: the thickened plasma membrane typical of cornified-

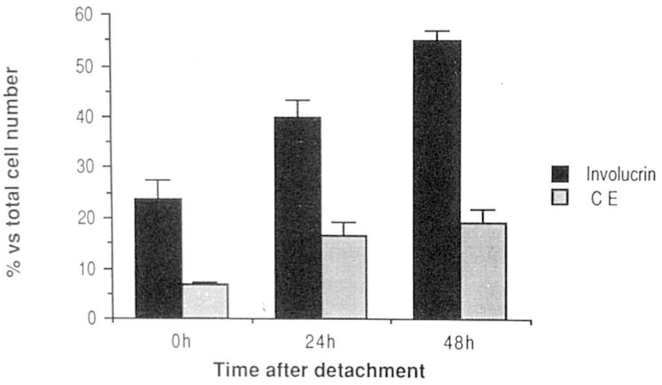

Fig. 6. Determination of the percentage of involucrin-positive cells and determination of the cornified envelope (CE) content after different storage periods following dispase detachment of keratinocyte cultures. Data are expressed as means ± SE of quadruplicate determinations.

envelope-containing keratinocytes was observed in cells which covered both sides of the detached cultures and the electrophoretic migration of newly synthesized proteins exhibited an enhanced production of some proteins in the detached cultures, one of them corresponding to involucrin (data not shown).

Lastly, in some preliminary attempts to inhibit the phenomenon of spatial reorganization, components of the extracellular matrix were added to the storage medium at a final protein concentration of 100 μg/ml. The tested substances (type I collagen, fibronectin, laminin or Matrigel (a laminin-rich preparation also containing type IV collagen and heparan sulfate proteoglycans: Nicosia and Ottinetti 1990) were unable to protect the initial polarity of the culture by preventing the spatial reorganization of keratinocytes. Fig. 8 illustrates the typical observations made 12 h after detachment in these conditions, but the spatial reorganization and the induction of terminal differentiation continue when the cells are studied for up to 48 h after detachment. We can further state that quantitative analysis of the proliferation and differentiation processes revealed no significant protection of the initial polarization when these proteins were added to the incubation media (data not shown). As a result of this absence of any protection of the culture's polarization, Fig. 8 also illustrates the good reproducibility obtained with multiple samples of dispase-detached cultures.

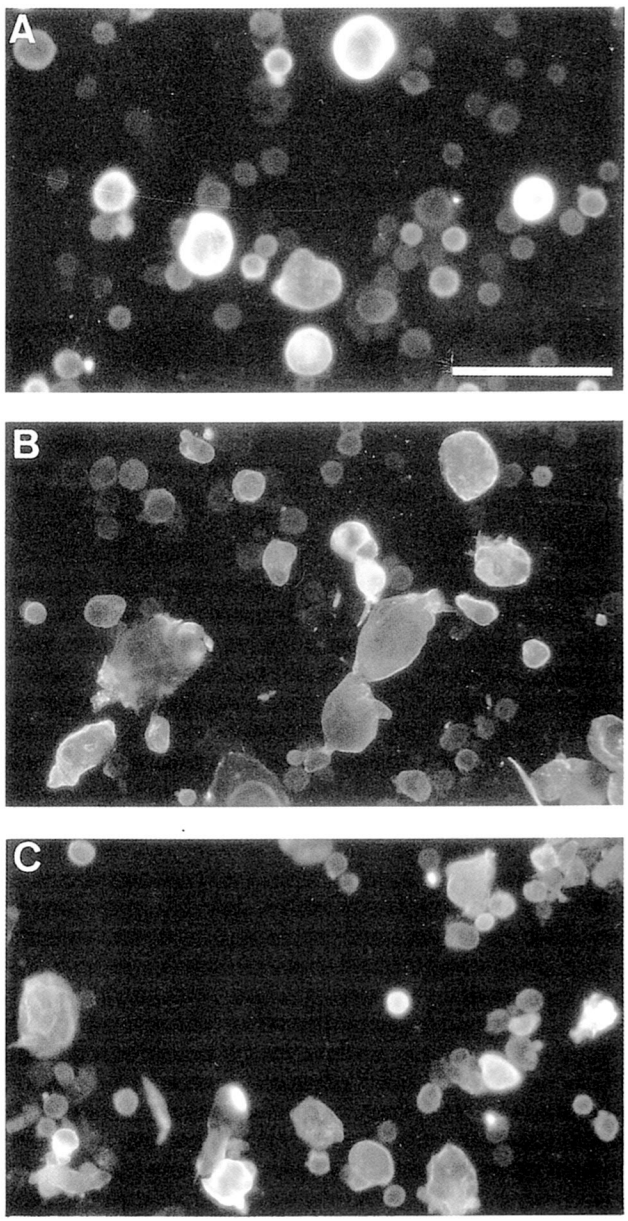

Fig. 7A–C. Immunofluorescent staining for involucrin. Keratinocytes were isolated by trypsinization immediately after detachment of dispase-detached cultures (**A**) or after storage for 24 h (**B**) or 48 h (**C**) at 37 °C in culture medium. They were then air-dried on coverslips and fixed with formaldehyde before fluorescein staining using a rabbit antiserum raised against involucrin. Scale bar represents 100 μm.

Discussion

Since we had previously studied the effects of storage on dispase-detached keratinocyte cultures with a view to their subsequent clinical utilization (Poumay et al. 1991; Boucher et al. 1991), we knew that phenomena linked to the control of the spatial organization of the epidermis and to its proliferation and differentiation occurred in these conditions. First, it was known that after the rapid contraction of cultures separated from the plastic surface, a well-known process originally described by Green et al. (1979), the contraction continued more slowly during the ensuing hours. Secondly, although we had observed that cell proliferation was progressively inhibited (in good agreement with the results obtained by others (Watt et al. 1988; Adams and Watt 1989) with suspended keratinocytes), the localization of cells that continued to proliferate was not yet strictly basal. Subsequently, the utilization of involucrin labelling highlighted our observations and revealed a complete spatial reorganization of keratinocytes in floating cultures, the cells sorting out into clusters. More precisely,

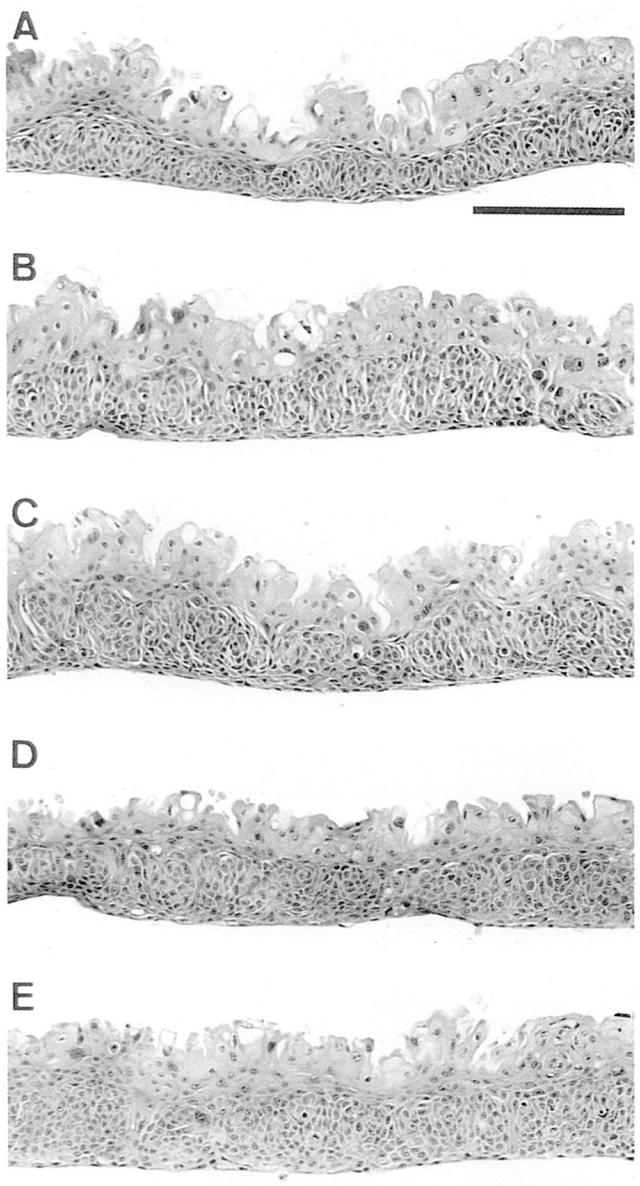

Fig. 8A–E. Dispase-detached epidermal sheets stored for 12 h at 37 °C in normal culture medium (**A**) or in the same medium supplemented with type I collagen (**B**), fibronectin (**C**), laminin (**D**) or Matrigel (**E**) before tissue fixation, embedding in paraffin, sectioning and staining with haemalum and erythrosin. The final concentration was 100 μg/ml for each component. Clustering is similar in each storage medium. Scale bar represents 200 μm.

14–16 h after detachment involucrin-positive keratinocytes were located at the periphery of clusters while involucrin-negative, occasionally DNA-synthesizing cells were found at the centre of clusters. Of particular interest is the great similarity of these observations with the sorting out of keratinocytes which results from the reaggregation of single-cell suspensions in an agarose pellet or in suspension. (It is especially relevant to compare, for example, Fig. 5B with figure 4b in the paper by Watt (1984). Our observations thus confirmed, with undissociated mixtures of keratinocytes (i.e. the dispase-detached cultures), Watt's (1987) hypothesis of a stronger cohesiveness in basal keratinocytes than in involucrin-positive cells, which could explain these unusual spatial organizations. However, in dispase-detached cultures this spatial reorganization into clusters requires specific cell-to-cell rearrangements rather than specific reaggregations of cells.

Could contracting forces in basal keratinocytes alone cause the appearance of involucrin-positive cells on the previous basal side? This seems unlikely for two reasons. First, after the rapid initial contraction achieved within 1 h (Fig. 2) the tissue is still highly polarized in superposed layers and looks as in Fig. 1A. Secondly, in recent experiments where the contraction of the culture was impeded by its association before detachment with a rigid support, we observed the same clustering phenomenon (F. Boucher and Y. Poumay unpublished data). This argues for the involvement of cell-to-cell spatial reorganization in this phenomenon.

In view of these results, how does an anchored epidermis remain polarized into superposed basal and suprabasal layers in vivo and in vitro? An answer can be suggested, using similar arguments. Predominant over the cohesiveness observed between basal cells is the adhesiveness of these cells to molecules of the extracellular matrix. Arguments for this assumption are the sorting out of keratinocytes after being injected into athymic mice (Lavker and Sun 1983) and the same sorting out observed after cells are encased in collagen (Watt 1987). Under these circumstances the polarity is the opposite of that observed in the absence of an adhesive substratum: the proliferating cells are located on the outside of the clusters, adhering to molecules of the extracellular matrix.

In epidermal cell–cell and cell–extracellular matrix interactions, the role of adhesive molecules of the integrin superfamily is of particular interest. These molecules are thought to be important in the development of the epidermis and in the maintenance of its spatial organization (De Luca et al. 1990; Hertle et al. 1991; Marchisio et al. 1991); some of them are involved in regulating the initiation of terminal differentiation (Adams and Watt 1989, 1990) and are subsequently under-expressed during terminal differentiation (Adams and Watt 1990; Nicholson and Watt 1991). Regarding a possible role of extracellular matrix receptors, we have shown that the addition of components of the extracellular matrix to floating epidermal sheets was unable to preserve the spatial organization of the epithelium. However, no binding of ligand to these receptors has yet been directly demonstrated. It follows that further investigations are needed to resolve this question, since we cannot rule out a proteolytic effect of dispase on membrane proteins. Nevertheless, we can note that dispase has a very selective action on type IV collagen and fibronectin (Stenn et al. 1989) and may add that following the detachment of cultures with dispase the integrins can still be detected, even on the basal side of basal cells, by immunocytochemistry (De Luca et al. 1990; Y. Poumay and F. Boucher, unpublished data).

We have also shown here that the equilibrium between the proliferating and the differentiating cell populations is perturbed in stored cultures after dispase detachment. Although the cell–cell interactions are preserved in these cultures, cell shape is rapidly modified when the floating sheets contract: in particular basal cells change from a flattened to a polygonal shape (Green et al. 1979; Banks-Schlegel and Green 1981). Simultaneously, cell proliferation is progressively inhibited in an irreversible way within 24–48 h after detachment and, furthermore, withdrawal from the cell cycle is accompanied by the induction of differentiation. These observations concur with those of others using suspended keratinocytes, the cell shape of which is also disturbed and thought to be responsible for the induction of differentiation; however, the effects observed in floating cultures are always slightly delayed compared with those observed in single-cell suspensions (Green 1977; Rheinwald 1979; Watt 1987; Watt et al. 1988; Adams and Watt 1989).

Modifications of the keratinocyte culture environment can also induce differentiation in floating sheets. It has been shown by ion-capture cytochemistry that a Ca^{2+} gradient exists in the epidermis in vivo (Menon et al. 1985). Basal keratinocytes proliferate in a low-calcium milieu whilst suprabasal layers are bathed in higher calcium concentrations. These environments in vitro are known to favour, respectively, keratinocyte proliferation (Hennings et al. 1980; Boyce and Ham 1983) and terminal differentiation (suprabasal keratins, filaggrin or involucrin synthesis (Yuspa et al. 1989; Pillai et al. 1990; Okumura et al. 1991), transcription of the loricrin gene (Hohl et al. 1991) and cornified envelope formation (Boyce and Ham 1983; Pillai et al. 1990)). If it is assumed that such a gradient, created by the adsorption of calcium to basement membrane components (Yuspa et al. 1989), is present in cultures of epidermal cells, detachment of the cultures would, unusually, allow identical high calcium concentrations to be present on both sides of the sheets, inhibiting cell proliferation and inducing differentiation. Regarding cell–cell interaction, one may ask whether the spatial reorganization might not be the result of a calcium-induced reorganization of junction components (Watt et al. 1984), which causes the migration of differentiating cells to the basal side of the culture. Accordingly, modulation of calcium concentrations, at the extracellular and/or intracellular levels, should induce changes in cell adhesion properties by the regulation of integrin activities in cell–extracellular matrix and cell–cell interactions (Kirchhofer et al. 1991; Marie et al. 1991). Looking for similarities with situations of epidermal detachment in vivo, for example in blisters, is certainly of great interest.

Lastly, it has been recognized that dispase-detached cultured keratinocyte sheets used as allografts favour wound healing, although cultured cells are not inserted into the renewed epidermis (De Luca et al. 1989; Hancock and Leigh 1989). This argues for the involvement of paracrine-type factors in these interactions between keratinocytes. The liberation of this kind of factor by dispase-detached cultures has yet to be demonstrated, but paracrine effects could be an explanation for the phenomena observed in our present study (Barrandon and Green 1987b; Coffey et al. 1987; Hancock and Leigh 1989; Bascom et al. 1989; Matsumoto et al. 1990).

In conclusion, the adhesion of basal cells to a substratum has been demonstrated to be crucial for the spatial organization of cultured keratinocytes into superposed layers. Moreover, it has been demonstrated that terminal differentiation is induced in non-adherent cultured sheets even though intercellular interactions are still present. Dispase-detached cultures seem, therefore, to constitute an interesting tool for studying the role of basal anchorage in keratinocyte physiology.

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